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Ischemic postconditioning confers cardioprotection and prevents reduction of Trx-1 in young mice, but not in middle-aged and old mice

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Abstract Thioredoxin-1 (Trx-1) is part of an antioxidant system that maintains the cell redox homeostasis but their role on ischemic postconditioning (PostC) is unknown. The aim of this work was to determine whether Trx-1 participates in the cardioprotective mechanism of PostC in young, middle-aged, and old mice. Male FVB young (Y: 3 month-old), middle-aged (MA: 12 month-old), and old (O: 20 month-old) mice were used. Langendorff-perfused hearts were subjected to 30 min of ischemia and 120 min of reperfusion (I/R group). After ischemia, we performed 6 cycles of R/I (10 s each) followed by 120 min of reperfusion (PostC group). We measured the infarct size (triphenyltetrazolium); Trx-1, total and phosphorylated Akt, and GSK3β expression (Western blot); and the GSH/GSSG ratio (HPLC). PostC reduced the infarct size in young mice (I/R-Y: 52.3 ± 2.4 vs. PostC-Y: 40.0 ± 1.9, $p < 0.05$), but this protection was abolished in the middle-aged and old mice groups. Trx-1 expression decreased after I/R, and the PostC prevented the protein degradation in young animals (I/R-Y: 1.05 ± 0.1 vs. PostC-Y: 0.52 ± .0.07, $p < 0.05$). These changes were accompanied by an improvement in the GSH/GSSG ratio (I/R-Y: 1.25 ± 0.30 vs. PostC-Y: 7.10 ± 2.10, $p < 0.05$). However, no changes were observed in the middle-aged and old groups. Cytosolic Akt and GSK3β phosphorylation increased in the PostC compared with the I/R group only in young animals. Our results suggest that PostC prevents Trx-1 degradation, decreasing oxidative stress and allowing the activation of Akt and GSK3β to exert its cardioprotective effect. This protection mechanism is not activated in middle-aged and old animals.

Keywords Ischemic postconditioning · Myocardial infarction · Aging · Thioredoxin-1

Introduction

Ischemic postconditioning (PostC) is an endogenous cardioprotection mechanism that reduces the infarct size [1–3]. Although ischemic preconditioning (IPC) significantly reduces myocardial injury, the applicability of IPC is limited by the unpredictable nature of ischemic events in clinical practice. In this sense, ischemic postconditioning is triggered during the clinically applicable time period of reperfusion, with the major limitation being the invasive protocol [4]. The importance of a reduced endothelial activation, neutrophil adherence, and consequently altered redox-sensible mechanisms in PostC was shown by Zhao et al. [1] in the original paper that described PostC. Furthermore, a reduction in superoxide anion generation [5], a decrease in nitric oxide (NO) production [6], and preservation of mitochondrial dysfunction [7] were also reported as mechanisms to reduce oxidative stress related with PostC cardioprotection. For these reasons, reduced reactive oxygen species (ROS) production in PostC [1, 8, 9] are in line with the idea that increased ROS production is implicated in the damage of myocardial reperfusion injury. Furthermore, PostC involves signal transduction pathways, which are similar to those seen in IPC [10]. In particular,

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autacoids such as adenosine, bradykinin, opioids, and also adrenergic receptors can trigger a complex protective signaling pathway including RISK pathway, mainly PI3K-Akt, MEK1/2, and GSK3β [11–13]. However, it is unclear whether the activation of this intracellular signaling pathway is linked to the changes evidenced in oxidative stress.

Thioredoxin-1 (Trx-1, 12KDa) is an endogenous system that regulates redox homeostasis and has cardioprotective effects against ischemia/reperfusion (I/R) injury [11–16]. In this sense, Aota et al. [14] demonstrated that the administration of recombinant human thioredoxin reduced the incidence of reperfusion arrhythmia. Similarly, Tao et al. [16] showed that administration of Trx-1 in vivo exerts significant protective effects on myocardial apoptosis decreasing myocardial infarct size, by inhibiting p38-MAPK activation. Thus, it is clear that Trx-1 has a protective effect against I/R injury. However, whether Trx-1 is involved in the PostC cardioprotection mechanism remains unknown. In this sense and to our knowledge, only Du et al. [17] showed that hydrogen sulfide postconditioning can enhance the cellular Trx system and play a protective role in I/R injury, but this study was only performed in the liver.

Moreover, it is widely known that I/R injury is exacerbated in old populations and that many of the protective mechanisms lose their effect with advanced age [18, 19]. But it is not clear whether this also occurs in middle-aged, when the deleterious effects of aging are already taking place. This stage of life has gained importance in recent decades because the onset of ischemic heart disease has decreased in recent years, probably due to increased risk factors associated to the Western lifestyle. Thus, a first objective was to study if PostC exerts its cardioprotective effect not only in young, but also in middle-aged and old animals. A second objective was to determine if the Trx-1 expression is involved in the cardioprotection conferred by PostC in these stages of life, and the possible changes occurring in the reduced/oxidized glutathione ratio (GSH/GSSG). Finally, a third objective was to study if Akt and GSK3β, RISK pathway proteins, are involved in the protection conferred by PostC and its relation with Trx-1.

Materials and methods

Animal care

All procedures performed in these studies involving animals were in accordance with the ethical standards of the Animal Care and Research Committee of the University of Buenos Aires (CICUAL UBA # 0037016/2012). FVB mice were housed in ventilated cages with a 12-h light/dark cycle and controlled temperature (20–22 °C), and fed with normal chow and water ad libitum.

Isolated mice hearts

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg body weight) and sodium heparin (500 UI/kg body weight). After ensuring sufficient depth of anesthesia, hearts were excised, and the aorta was immediately cannulated with a 21-gage cannula. Afterward, hearts were perfused according to the Langendorff technique at constant flow with Krebs medium [118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.5 mM CaCl2, 24.8 mM NaHCO3, 10 mM glucose (pH 7.4)] equilibrated with 95 % O_2 and 5 % CO_2 at 37 °C. A small latex fluid-filled balloon was connected via a thin plastic catheter (P50) to a Deltran II pressure transducer (Utah Medical System, UT, USA), and was inserted into the left ventricle via the left atrium. The catheter with the transducer was positioned in such a way that it secured the position of the balloon in the left ventricle. Two electrodes were sutured and connected to a pacemaker in order to induce a constant heart rate of 473 ± 30 beats/min. In order to record coronary perfusion pressure (CPP), a second pressure transducer was connected to the perfusion line. Hearts were perfused at constant flow in order to obtain a CPP around 70 mmHg during the initial stabilization period and then maintained constant throughout the experiment. The coronary flow was constant during all the experimental procedures (I/R-Y: 3.8 ± 0.2 ml; PostC-Y; 4.1 ± 0.5 ml; I/R-MA: 4.2 ± 0.4 ml; PostC-MA: 4.0 ± 0.5 ml; I/R:-O 4.3 ± 0.3 ml; PostC-O: 3.9 ± 0.5 ml). Left ventricular developed pressure (LVDP) and the maximum rate of rise of left ventricular pressure (LV + dP/dt_{max}) were used as contractile state indexes. Left ventricular end diastolic pressure (LVEDP), a myocardial stiffness index in the isovolumic heart, was also measured.

Experimental protocols and groups

Ischemia/reperfusion protocol: Myocardial infarction was induced by 30 min of global no-flow ischemia followed by 120 min of reperfusion. Global no-flow ischemia was induced by abruptly decreasing the total coronary flow provided by the perfusion pump.

Ischemic postconditioning protocol: The same protocol as in I/R group was repeated, but at the onset of reperfusion we performed 6 cycles of 10 s of reperfusion followed by 10 s of ischemia (2 min total intervention, ischemic PostC protocol). Reperfusion was then continued for 120 min.

Normoxic protocol (n = 5 for each age stage): This group was only used as a control group for the Western blot and glutathione determinations. Hearts were perfused for

30 min in normoxic conditions, and the left ventricle (LV) samples were taken for their further processing.

Young (3 month-old), middle-aged (12 month-old), and old (20 month-old) male mice hearts were used and divided into six experimental groups: (1) Ischemia/reperfusion young (I/R-Y, $n = 8$); (2) Ischemic postconditioning young (PostC-Y, $n = 7$); (3) Ischemia/reperfusion middle-aged (I/R-MA, $n = 8$); (4) Ischemic postconditioning middle-aged (PostC-MA, $n = 9$); (5) Ischemia/reperfusion old (I/R-O, $n = 8$); and (6) Ischemic postconditioning old (PostC-O, $n = 7$). For the middle-aged group, mice should be at least 10 month-old; the upper age limit for the middle-aged group is typically 14–15 month-old [20, 21]. For this reason we decided to use 12 month-old as middle-aged mice, and 20 month-old as old mice.

All the experimental groups were repeated and LV samples were taken at 15 min of reperfusion, after 30 min of global ischemia, for Western blot and glutathione determinations.

Infarct size measurement

The assessment of the infarct size was performed using 2, 3, 5-triphenyltetrazolium chloride (TTC). After 120 min of reperfusion, the hearts were frozen and cut into 1 mm transverse slices from apex to base. Sections were incubated for 20 min in 1 % TTC (pH 7.4, 37 °C) and then immersed in 10 % formalin. With this technique, viable sections were stained red, while the non-stained sections corresponded to the infarct area. Sections were traced to acetate sheets and planimetered (Image Pro Plus, version 4.5). Infarct size was expressed as a percentage of the left ventricular area.

Samples preparation

Tissue homogenates Tissue samples (0.2 g of wet weight) were homogenized with a glass-Teflon homogenizer in a medium consisting of 120 mM KCl and 30 mM phosphate buffer (pH 7.4) (1:5) at 0–4 °C. The suspension was centrifuged at 600 g for 10 min at 4 °C to remove nuclei and cell debris. The pellet was discarded, and the supernatant was used as "homogenate" [22].

Reduced (GSH) and oxidized (GSSG) glutathione levels

Heart samples were homogenized with a glass-Teflon homogenizer in solution containing 1 M HClO, 4–2 mM EDTA (1:1), and centrifuged at 16,000 g for 20 min at 4 °C. Supernatants were filtered through 0.22 μm cellulose acetate membranes (Corning Inc., NY, US), and frozen at −80 °C until use. HPLC analysis was performed in a Perkin Elmer LC 250 liquid chromatography (Perkin Elmer, Waltham, MA, US), equipped with a Perkin Elmer LC ISS 200 advanced sample processor, and a Coulochem II (ESA, Bedford, MA, US) electrochemical detector. A Supelcosil LC-18 (250 × 4.6 mm ID, 5 μm particle size) column protected by a Supelguard (20 × 4.6 mm ID) precolumn (Supelco, Bellfonte, PA, US) was used for sample separation. GSH and GSSG were eluted at a flow rate of 1.2 mL/min with 20 mM sodium phosphate (pH 2.7), and electrochemically detected at an applied oxidation potential of +0.800 V. Results were expressed as μM [23].

Western blot

We performed additional experiments to obtain heart samples ($n = 5$ in each group) for Western blot analysis at 15 min of reperfusion. The selected time is in concordance with previous studies showing that the activation of proteins involved in cell survival (RISK pathway) occurs at early stages of reperfusion [11, 24–26]. Heart tissue was homogenized in ice for approximately 2 min with extraction buffer (pH 7.4), composed of: Tris 1.2 mM, NaCl 0.36 mM, sodium dodecyl sulfate (SDS) 0.1 %, Triton 1 %, DTT 0.2 mM, protease and phosphatase inhibitors cocktail (Thermo Scientific) at a rate of 500 μL buffer every 150 mg of tissue using a PRO 200 Scientific INC homogenizer. Subsequently, homogenates were centrifuged at 12,608×*g* during 20 min at 4 °C. The supernatant protein concentration was quantified with the Bradford method.

Trx-1, Akt/pAkt, and GSK3β/pGSK3β expression

After protein quantification, 50 μg of each sample was separated by 16 % Tricine-SDS-PAGE gels (for Trx-1 expression), and by 12 % Glycine-SDS-PAGE gels (for Akt,/pAkt, and GSK3β/pGSK3β) and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Scientific) that was later blocked with 5 % BSA for two hours at room temperature. Next, the membrane was incubated with anti-Trx-1 (1:1000) (Cell Signaling), anti-phospho-Akt for Ser473 residue (1:1000) (Cell Signaling), and anti-phospho-GSK3β for Ser9 residue (1:1000) (Cell Signaling) overnight at 4 °C with agitation. It was later incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP, 1:15000) (Millipore) for an hour at room temperature. The membrane was developed with photographic plates (Kodak) and Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Trx-1 expression was quantified by densitometry with Image Gauge 4.0 software (Fujifilm) compared to the charge control values, and anti-GAPDH (1:1000, Cell Signaling), measured in the same

membranes, was used as loading control. We also quantified p-Akt and p-GSK3ß by densitometry, and we used anti GAPDH (1:1000, Cell Signalling) as loading control measured in the same membranes.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Inter-group comparisons were performed using analysis of variance, followed by Bonferroni test for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Table 1 shows the systolic function behavior at baseline and during different reperfusion times. In all groups, LVDP (mmHg) and LV $+dP/dt_{max}$ (mmHg/s) were significantly lower compared to pre-ischemic values, but showed no significant differences among groups throughout the procedure. LVEDP (mmHg) increased at 30 min compared to pre-ischemic values without differences among the groups.

Figure 1 shows the behavior of the infarct size in all experimental groups. In young animals, as expected, PostC reduced the infarct size (I/R-Y: 52.3 ± 2.4 vs. PostC- Y: 40.0 ± 1.9 %, $p < 0.05$). However, both in middle-aged (I/R-MA: 51.8 ± 2.6 vs. PostC-MA: 50.3 ± 5.1 %), and in old animals (I/R-O: 63.8 ± 5.7 vs. PostC-O: 59.6 ± 5.2 %), the protection conferred by the PostC was abolished. Panel B shows representative slices of the different experimental groups.

The expression of pAkt (Ser^{473}) normalized by the total Akt and GAPDH in young (Panel A), middle-aged (Panel B), and old mice (Panel C), can be observed in Fig. 2. An increase in Akt phosphorylation can be observed in the PostC-Y group (Nx-Y: 1.00 ± 0.10 and I/R-Y: 0.98 ± 0.17 vs. PostC-Y: 1.59 ± 0.10 AU, $p < 0.05$). However, this increased phosphorylation was not observed neither in middle-aged (Nx-MA: 0.94 ± 0.06, I/R-MA: 0.81 ± 0.04 and PostC-MA: 0.79 ± 0.11 AU) nor in old mice (Nx-O: 0.99 ± 0.11, I/R-O: 0.87 ± 0.03, PostC-O: 1.12 ± 0.22 AU).

In Fig. 3, the expression of pGSK3β (Ser^{9}) normalized by the total GSK3β and GAPDH can be observed in young (Panel A), middle-aged (Panel B), and old mice (Panel C). A similar behavior is observed among the groups. An increased GSK3β phosphorylation was only observed in the PostC-Y group (Nx-Y: 0.94 ± 0.37 and I/R-Y: 1.60 ± 0.53 vs. 3.89 ± 0.52 AU, $p < 0.05$) without changes in neither the middle-aged (Nx-MA: 1.00 ± 0.06,

Table 1 Left ventricular function

	LVDP (mmHg)	LVEDP (mmHg)	LV + dp/dt_{max} (mmHg/sec)	CPP (mmHg)
I/R				
Baseline				
Young	89.0 ± 5.0	7.0 ± 1.0	2904 ± 278	78.2 ± 2.6
Middle-aged	91.0 ± 8.0	8.6 ± 2.8	3047 ± 273	72.8 ± 3.2
Old	84.4 ± 2.5	7.5 ± 1.2	2848 ± 129	77.6 ± 7.8
30′R				
Young	16.0 ± 3.0*	24.0 ± 5.0*	371 ± 34*	111.0 ± 10.7*
Middle-aged	21.2 ± 2.6*	30.9 ± 5.9*	436 ± 22*	103.1 ± 5.8*
Old	17.1 ± 0.2*	21.6 ± 4.8*	394 ± 55*	123.7 ± 2.6*
PostC				
Baseline				
Young	88.5 ± 3.8	8.5 ± 1.5	2775 ± 117	74.8 ± 2.5
Middle-aged	92.9 ± 8.7	7.6 ± 1.7	3111 ± 199	75.1 ± 4.4
Old	94.0 ± 10.0	7.0 ± 0.1	3239 ± 386	75.0 ± 11.0
30′R				
Young	24.1 ± 4.7*	42.3 ± 9.8*	461 ± 155*	98.0 ± 6.5*
Middle-aged	31.3 ± 4.3*	39.6 ± 8.3*	601 ± 225*	109.9 ± 16.3*
Old	28.2 ± 3.8*	40.6 ± 4.6*	595 ± 477*	127.3 ± 7.2*

Left ventricular developed pressure (LVDP, mmHg), left ventricular end diastolic pressure (LVEDP, mmHg), maximal rate of rise of left ventricular pressure (LV + dP/dt_{max}), and coronary perfusion pressure (CPP). I/R: ischemia/reperfusion, PostC: postconditioning, Baseline: pre-ischemic values, 30́R: 30 min of reperfusion

* $p < 0.05$ vs. Baseline

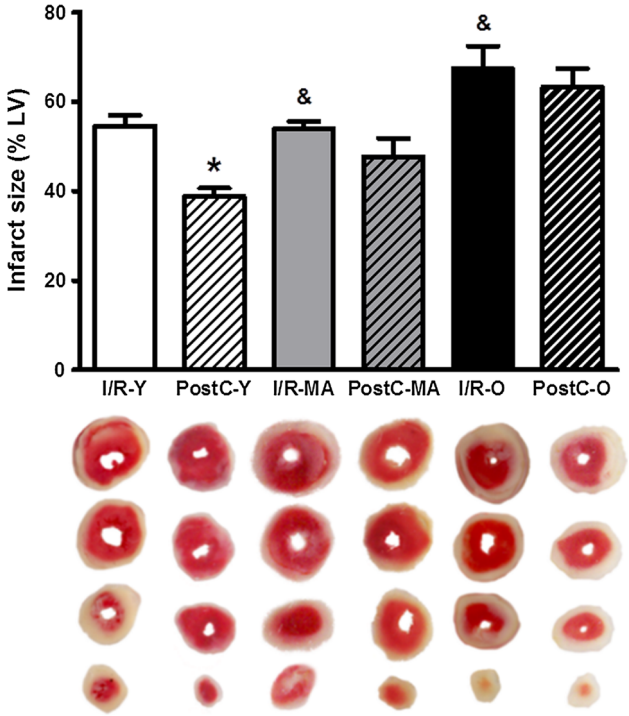

Fig. 1 Infarct size expressed as a percentage of the total left ventricular area. Infarct size decreased significantly in PostC-Y group but this cardioprotective effect was abolished in middle-aged and old groups. *Panel B* shows representative slices of the different experimental groups. $^{*}p < 0.05$ vs. I/R-Y; $^{\&}p < 0.05$ vs. PostC-Y. *I/R-Y* wild type in young animals (3 month-old), *I/R-MA* wild type in middle-aged animals (12 month-old), *I/R-O* wild type in old animals (20 month-old), *PostC-Y* wild type in young animals, *PostC-MA* wild type in middle-aged animals, *PostC-O* wild type in old animals

I/R-MA: 0.97 ± 0.06, PostC-MA: 0.94 ± 0.01 AU) nor in the old animals (Nx-O: 1.00 ± 0.12, I/R-O: 1.25 ± 0.03, PostC-O: 1.15 ± 0.11 AU).

Figure 4 shows the behavior of oxidative stress in the different young mice protocols, since we only detected protection in PostC-Y mice. We measured the oxidized (GSH) and reduced (GSSG) glutathione concentration (μg/g of tissue) and the ratio between them. In Panel A, it can be observed that the GSH concentration significantly reduces after I/R in the young animals (Nx-Y: 33.52 ± 1.96 vs. I/R-Y: 23.64 ± 1.43, $p < 0.05$) returning to its pre-ischemic values in the PostC-Y (32.03 ± 2.40, $p < 0.05$ vs. I/R-Y). In Panel B, it can be observed that the GSSG concentration significantly increases in the I/R (Nx-Y: 8.31 ± 2.86 vs. I/R-Y: 29.3 ± 4.8, $p < 0.05$) decreasing when PostC was performed, reaching values similar to the normoxic levels (9.82 ± 3.28 vs. I/R-Y, $p < 0.05$). Finally, in Panel C, the GSH/GSSG ratio can be observed, a reliable indicator of redox status. After I/R, the GSH/GSSG ratio decreases (I/R-Y: 1.25 ± 0.30 vs. Nx-Y: 12.43 ± 0.97, $p < 0.05$). PostC improves such ratio reaching values similar to the pre-ischemic ones (7.10 ± 2.10 vs. I/R-Y, $p < 0.05$).

Figure 5 shows Trx-1 expression in young, middle-aged, and old mice. A decrease of Trx-1 expression was observed in I/R-Y group (Nx-Y: 1.16 ± 0.09 vs. I/R-Y: 0.60 ± 0.08 AU, $p < 0.05$), but PostC avoids Trx-1 degradation after I/R protocol (PostC-Y: 0.94 ± 0.10 AU vs. I/R-Y, $p < 0.05$) (Panel A). In both, in middle-aged and old mice, PostC protocol did not preserve Trx-1 expression after I/R (I/R-EM: 0.54 ± 0.12, PostC-EM: 0.63 ± 0.08 and I/R-O: 0.38 ± 0.08, PostC-O: 0.49 ± 0.10 AU) (Panel B and C, respectively). Regarding Trx-1 expression in normoxic condition, we detected that in middle-aged and old mice, Trx-1 levels were lesser than in young mice (Nx-Y: 1.1 ± 0.1 vs. Nx-MA: 0.42 ± 0.05 and Nx-O: 0.43 ± 0.08 AU, $p < 0.05$) (Fig. 6).

Discussion

Our results demonstrate that PostC decreases the infarct size in young mice, as we expected, but this cardioprotection was abolished in middle-aged and old mice. The reduction of infarct size in PostC are according with Trx-1 levels preservation and an improvement in the GSH/GSSG ratio, a reliable indicator of redox status, in comparison to I/R group. Also, this cardioprotection was accompanied by the Akt activation and the phosphorylation and inhibition of GSK3β, both proteins related to the cell survival pathway. Therefore, our data suggest that the preservation of Trx-1 levels in young mice subjected to a PostC protocol would reduce oxidative damage and allow the RISK signaling pathway (Akt/GSK3β) activation to increase cell survival. In accordance with the lack of cardioprotection in aging, we also detected a decrease in Trx-1 expression in normoxic conditions in middle-aged and old mice compared with young mice.

It is known that Trx-1 has a beneficial effect on the infarct size against ischemia/reperfusion injury [16, 27]. Tao et al. [16] demonstrated that Trx has a protective effect on infarct size and apoptosis by reducing ischemia/reperfusion-induced oxidative/nitrative stress in a mice model that received intraperitoneal recombinant human Trx administration. Regarding Akt/pAkt in particular, Adluri et al. [28] demonstrated that Trx-1 overexpression induces an Akt-signaling pathway compared to wild-type mice during ischemic stress, and this could be related to a reduction in oxidative stress. These authors used a chronic model of myocardial infarction and showed that the Akt was phosphorylated several days after the ischemic insult. However, our research showed that Akt was the probable target for Trx to provide acute protection in an I/R in vitro model. Thus, while it is clear that Trx-1 decreases the

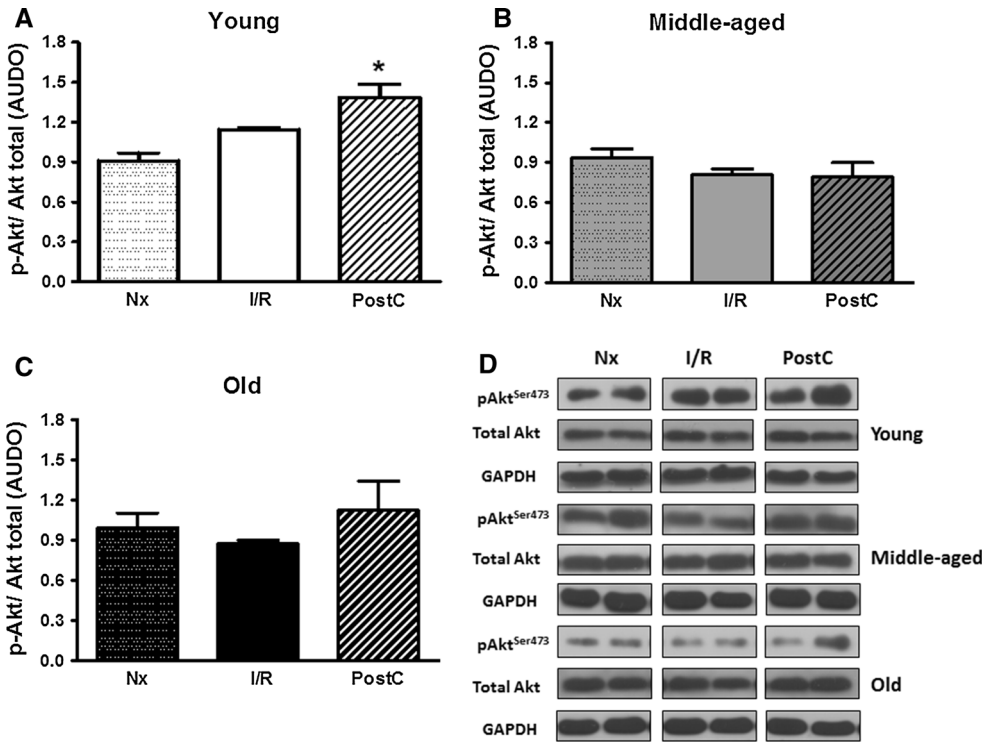

Fig. 2 Akt phosphorylation (Ser^{473}) protein expression in young (**a**), middle-aged (**b**), and old animals (**c**). We used normoxic group (Nx), ischemia/reperfusion group (I/R) and ischemic postconditioning group (PostC). There were not significant changes in Akt phosphorylation (Ser^{473}) protein expression in middle-aged and old groups. However the PostC-Y group presents a significantly enhanced Akt phosphorylation. There were no significant changes in the cytosolic Akt and GAPDH protein expression. **d** Representative blots. $^{*}p < 0.05$ vs. Nx. *Nx-Y* normoxic heart in young animals (3 month-old); *Nx-MA* normoxic hearts in middle-aged animals (12 month-old); *Nx-O* normoxic heart in old animals (20 month-old); *I/R-Y* ischemia/reperfusion in young animals; *I/R-MA* ischemia/reperfusion in middle-aged animals; *I/R-O* ischemia/reperfusion in old animals; *PostC-Y* postconditioning in young animals; *PostC-MA* postconditioning in middle-aged animals; *PostC-O* postconditioning in old animals

injury by I/R, there are no studies that have evidenced that Trx-1 is involved in the endogenous acute myocardial protective mechanisms such as postconditioning. In this sense, Turoczi et al. [27] demonstrated that reperfusion of ischemic myocardium resulted in the downregulation of Trx-1 expression, which was up-regulated in the adapted myocardium subjected to an ischemic preconditioning protocol. The administration of cis-diamminedichloroplatinum, an inhibitor of Trx-1, completely abolished the cardioprotection afforded by ischemic preconditioning. However, there are no papers that have studied whether Trx-1 participates in the cardioprotection conferred by PostC. In this sense, only Du et al. [17] demonstrated that hydrogen sulfide postconditioning increases Trx activity and Trx-1 protein expression, and decreases TXNIP protein expression compared to the group subjected to I/R. However, this study was performed in rat livers. Therefore, our study is the first one to demonstrate that Trx-1 would participate in the myocardial protection conferred by PostC. The myocardial infarction of the young mice group decreased by approximately 23 %. This reduction looks modest, but it is important to mention that some degree of variability is present in the PostC protection magnitude, even while using the same experimental model, between the different published studies [29–31].

It is also known that certain comorbidities, such as age, interfere in the cardioprotection mechanism [18, 32, 33]. When the aged-heart is exposed to different stress stimuli, myocardial damage is exacerbated and a further deterioration of the myocardial function and a lower tolerance to the ischemic injury occur [34, 35]. This loss of tolerance begins during the middle-aged of life (12 month-old) and it becomes more manifest during aging (18 month-old and 24-28 month-old) [21]. In this sense, Zhang et al. [36] demonstrated that the activity of Trx was significantly reduced in aging hearts even before they were subjected to myocardial ischemia/reperfusion. For these reasons, infarct size was larger in aged C57/BI6 mice (20 month-old). These data are in concordance with our findings showing a decrease in Trx-1 in normoxic conditions in middle-aged and old mice, compared with young mice. In a similar manner, Azhar et al. [37] demonstrated an increase in the infarct size in C57/BI6 mice at 22–24 month-old, subjected to 45 min of ischemia and 4 h of reperfusion. However,

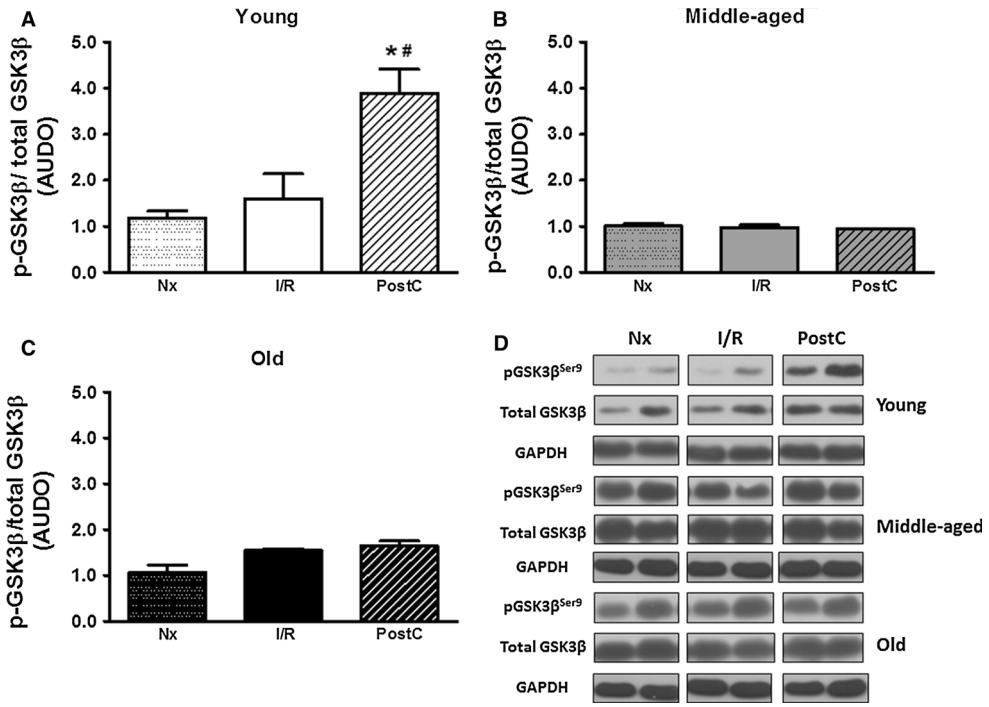

Fig. 3 GSK3β phosphorylation (Ser^9) protein expression in young (**a**), middle-aged (**b**), and old animals (**c**) in normoxic (Nx), ischemia/reperfusion (I/R) and postconditioning (PostC) protocols. There were no significant changes in GSK3β phosphorylation (Ser^9) protein expression in middle-aged and old groups; however, in PostC-Y group present a significantly enhanced GSK3β phosphorylation. There were no significant changes in the cytosolic GSK3β and GAPDH protein expression. **d** Representative blots. $^*p < 0.05$ vs. Nx; $^\#p < 0.05$ vs. I/R. *Nx-Y* normoxic heart in young animals (3 month-old); *Nx-MA* normoxic heart in middle-aged animals (12 month-old); *Nx-O* normoxic heart in old animals (20 month-old); *I/R-Y* ischemia/reperfusion in young animals; *I/R-MA* ischemia/reperfusion in middle-aged animals; *I/R-O* ischemia/reperfusion in old animals; *PostC-Y* postconditioning in young animals; *PostC-MA* postconditioning in middle-aged animals; *PostC-O* postconditioning in old animals

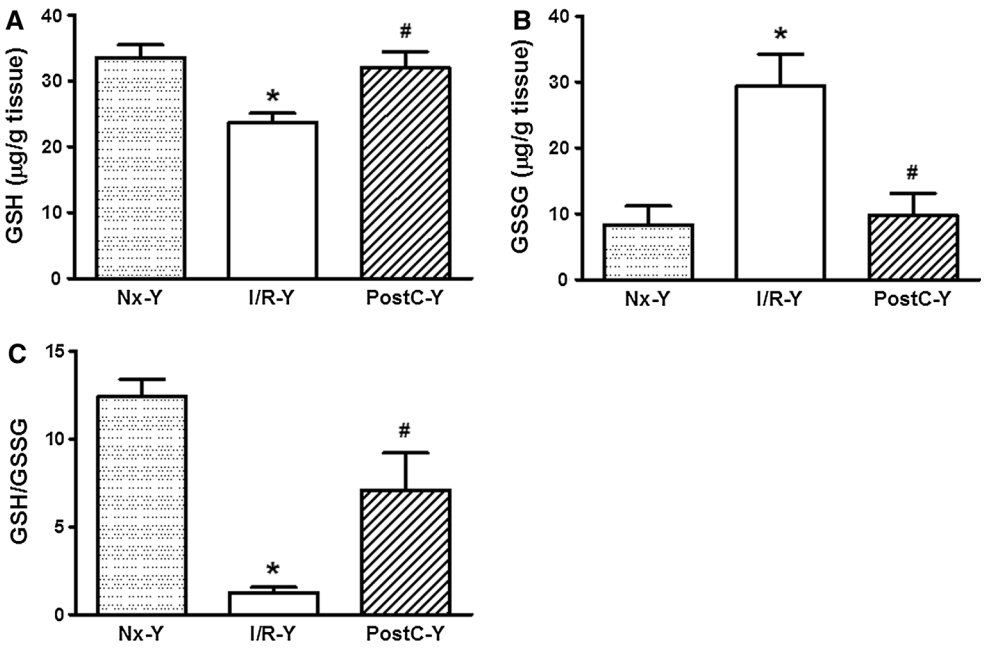

Fig. 4 Oxidative stress. GSH (**a**), GSSG (**b**), and GSH/GSSG ratio (**c**). There was a significant increase in oxidative stress in the I/R-Y group. The GSH levels were decreased and the GSSG levels were increased compared to Nx hearts. This imbalance in redox homeostasis returned to pre-ischemic values after postconditioning protocol $^*p < 0.05$ vs. Nx; $^\#p < 0.05$ vs. I/R. *Nx-Y* normoxic hearts in young animals (3 month-old); *I/R-Y* ischemia/reperfusion in young animals; *PostC-Y* ischemic postconditioning in young animals

this reduction of tolerance to ischemia was not evidenced in the infarct size of C57/BI6 mice at 13 month-old subjected to 30 min of ischemia and 2 h of reperfusion [35]. Taking together all the previous papers, it is clear that when the aged heart is exposed to different types of stress, such as ischemia, an amplification of damage occurs, i.e., a

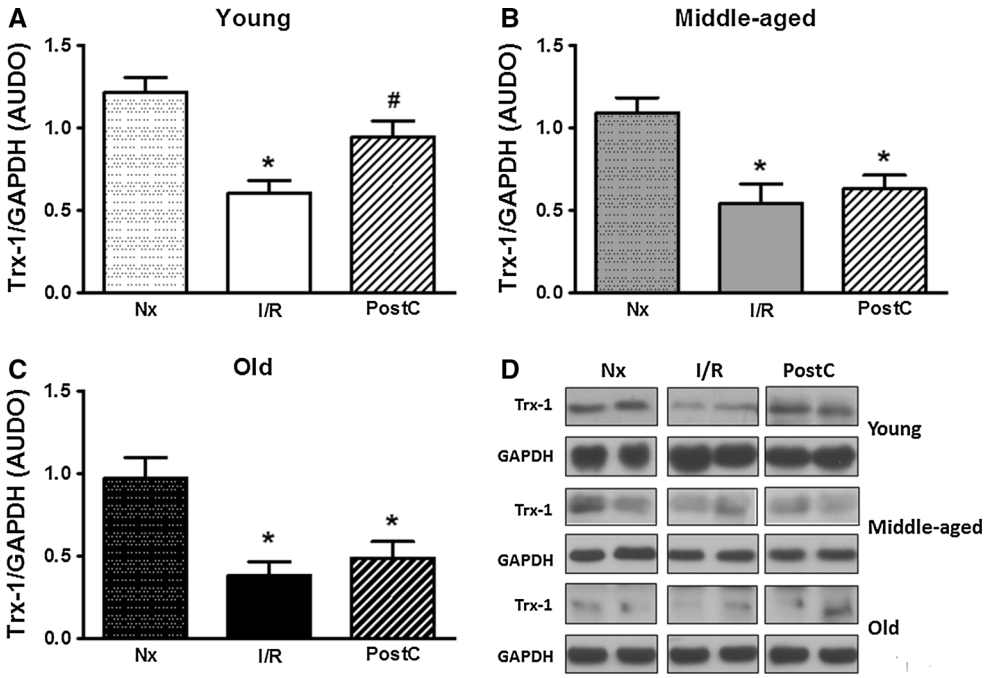

Fig. 5 Trx-1 expression in young (**a**), middle-aged (**b**), and old animals (**c**) in normoxic (Nx), ischemia/reperfusion (I/R), and postconditioning (PostC) protocols. **a** I/R decreases Trx-1 levels, and PostC preserves the protein content. There were no significant changes in Trx-1 expression in middle-aged and old groups, between I/R and PostC-Y groups. **d** Representative blots. $^{*}p < 0.05$ vs. Nx; $^{\#}p < 0.05$ vs. I/R. *Nx-Y* normoxic hearts in young animals (3 month-old); *Nx-MA* normoxic hearts in middle-aged animals (12 month-old); *Nx-O* normoxic hearts in old animals (20 month-old); *I/R-Y* ischemia/reperfusion in young animals; I/R-MA: ischemia/reperfusion in middle-aged animals; *I/R-O* ischemia/reperfusion in old animals; *PostC-Y* postconditioning in young animals; *PostC-MA* Postconditioning in middle-aged animals; *PostC-O* Postconditioning in old animals

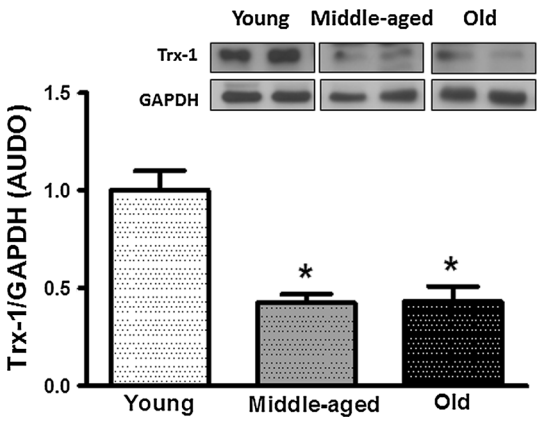

Fig. 6 Trx-1 expression in normoxic (Nx) condition among young, middle-aged, and old mice. In middle-aged and old mice, Trx-1 expression decreases compared with young animals. $^{*}p < 0.05$ vs. young mice

further deterioration of cardiomyocytes, the tolerance to ischemic injury is reduced, and for these reasons the infarct size is larger [18, 27, 32, 35, 37]. These findings are in accordance with our results, since we evidenced an increase in the infarct size only in old mice (20 month-old) but we did not observe changes in the infarct size in middle-aged mice (12 month-old).

In agreement with previous studies using mice isolated heart [38] in our study, PostC in young mice reduced infarct size but was unable to improve ventricular function (contractile state and myocardial stiffness).The absence of improvement of LV function in our study may be due to the presence of myocardial stunning areas peripheral to the infarct zone [38, 39]. In this sense, it has been well shown that the presence of a certain degree of post-ischemic dysfunction (stunned myocardium) reverses approximately after 48/72 h of reperfusion; therefore, the change in infarct size in acute experiments does not significantly influence the ventricular function [39].

Although it is accepted that Akt and GSK3β phosphorylation decreases after an I/R protocol, in concordance with our findings, several authors also show that after an I/R protocol, the phosphorylation of these proteins related to pro-survival pathway does not decrease in comparison to their pre-ischemic values [40–43]. However, it is important to mention that the Akt/GSK3β complex activation occurred during reperfusion after performing PostC in young mice. A limitation of our study is that although PostC increased phosphorylation of Akt/GSK3β, these results do not provide direct cause and effect relationship because no attempt was made to either inhibit or use knockout mice for these proteins.

In summary, our results strongly suggest that PostC decreases the infarct size in young mice due to a preservation of Trx-1 levels after I/R. This preservation of Trx-1 levels, as expected, showed an improvement in the tissue redox status, evidenced through the GSH/GSSG ratio in young animals. This cardioprotection was accompanied by Akt activation and the phosphorylation and inhibition of GSK3β, both proteins related to the cell survival pathway. However, the protection conferred by PostC was abolished in middle-aged and old animals, suggesting that the changes that can occur with aging are able to modify the behavior of the endogenous antioxidant systems and therefore abolish PostC cardioprotection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal rights statement All procedures performed in these studies involving animals were in accordance with the ethical standards of the Animal Care and Research Committee of the University of Buenos Aires (CICUAL UBA # 0037016/2012).

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